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Genomes of evolutionarily divergent members of the human T-cell leukemia virus family (HTLV-I and HTLV-II) are highly conserved, especially in pX

(human retrovirus/adult T-cell leukemia/lymphoma/acquired immunodeficiency syndrome)

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ABSTRACT **Human T-cell leukemia virus (HTLV) is a family of related human T-lymphotropic retroviruses closely linked with certain human T-cell malignancies and associated with many cases of acquired immunodeficiency syndrome (AIDS). We isolated and molecularly cloned HTLV from patients with both types of clinical disorders and found by restriction endonuclease mapping and core and envelope protein analysis that at least two evolutionarily divergent viral subgroups exist, HTLV-I and HTLV-II. Previous studies have failed to detect significant nucleotide sequence homology between HTLV-I and HTLV-II even though these different members of the HTLV family share certain biologic properties such as T-cell tropism and transformation. To further test these viruses for conserved regions in their genomes, we examined hybridization between HTLV-I and HTLV-II by using Southern blotting and heteroduplex mapping at different melting points. These two techniques produced similar results, showing that HTLV-I and HTLV-II proviruses have, in fact, strongly conserved nucleotide sequences in the pX region and lesser although still substantial homology in the LTR, *gag*, *pol*, and *env* regions. These data provide experimental evidence that HTLV-II, like HTLV-I, contains pX sequences. Although the function of pX is unknown, its conservation in evolutionarily divergent human T-lymphotropic viruses implies a biologically important function. It is possible, but unproven, that pX could encode proteins involved in T-cell tropism, cell transformation, immune suppression, or other biologic actions characteristic of the HTLV family.**

The discovery of a human retrovirus, human T-cell leukemia virus (HTLV), generated widespread scientific interest (1, 2). In addition, its unique biological properties, particularly its T-cell tropism and close association with certain human malignancies, further stimulated interest in this virus (for review, see ref. 3). HTLV was isolated and characterized in patients with aggressive T-cell malignancies from the United States (1, 2) and in patients from Japan, Israel, the Caribbean, Africa, and South America (refs. 3–9; unpublished observations). More recently, evidence for HTLV infection in some patients with the acquired immunodeficiency syndrome (AIDS) has been found (10–12). Many viral isolates from patients with different clinical disorders and from different geographic regions have been analyzed in regard to their genomic organization and protein structure. With the exception of two isolates, HTLV viruses associated with T-cell malignancies have been found to be very similar and therefore have been identified as HTLV-I (3, 5–7, 13, 14). The two viral isolates that have been sufficiently characterized to recognize significant differences from HTLV-I are designated HTLV-Ib and HTLV-II. HTLV-Ib, obtained from an African man with T-cell lymphoma, is highly homologous to HTLV-I by molecular hybridization but contains a short region of restriction enzyme site divergence in the envelope-pX region (15). HTLV-II, isolated from a patient with a T-cell variant of hairy cell leukemia (16), is more distantly related to HTLV-I as determined by molecular hybridization and core and envelope protein analysis (16–18). A second HTLV-II isolate has been obtained from a patient with AIDS (ref. 15; unpublished results). Despite differences in their genomic structure and core and envelope proteins, it is clear that all HTLV isolates so far identified are related members of the same family of viruses.

Because all known HTLVs share certain biological features, such as T-cell tropism (19, 20) and the ability to transform primary T cells *in vitro* (4–6, 8, 21–23), and because they have all been associated with clinical disorders involving mature T lymphocytes (3), we hypothesized that different HTLV subgroups could share certain regions of nucleic acid homology, which in turn are responsible for their similar biologic activities. To study this possibility, we employed Southern blot hybridization and heteroduplex mapping at different melting points (t_m) to examine the cloned proviral genomes of HTLV-I, HTLV-Ib, and HTLV-II for regions of conserved nucleic acid sequences. In this paper, we show that a region (pX), so far unique to the HTLV family and of unknown function (24), is highly conserved in all known isolates of HTLV and that the remainder of the genome, including the long terminal repeat (LTR), *gag*, *pol*, and *env* regions, is conserved to a lesser although still substantial degree.

MATERIALS AND METHODS

Molecular Cloning of HTLV Proviruses. The molecular cloning of complete genomes of prototype HTLV-I (λ23-3 and λCH-1), HTLV-II (λMO15A and λJP-1), and HTLV-Ib (λMC-1) proviruses has been described (15, 17, 25).

Southern Blot Hybridization. Regions of nucleotide sequence homology between HTLV-II and HTLV-I/HTLV-Ib were examined by Southern blot hybridization under conditions of varied stringency (26–28). Restriction enzymes cutting at selected points within the proviral genomes were chosen so as to distinguish hybridization of the probes to fragments from LTR, *gag*, *pol*, *env*, and pX regions of the HTLV genomes. ^{32}P-labeled probes used in this study included inserts containing the entire HTLV-II genome (λMO15Ai and pMO-4i), the 3′ *Bam*HI/*Bam*HI fragment of HTLV-II (pMO-3i), and the entire HTLV-I *Sst* I/*Sst* I insert

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Abbreviations: HTLV, human T-cell leukemia virus; LTR, long terminal repeat; kb, kilobase(s); kbp, kilobase pair(s).

derived from λ23-3 and subcloned in pBR322 (p23-3i).

Two approaches were used to vary the stringency of heteroduplex formation. The first method was to hybridize probe to blotted DNA fragments at relatively low stringency (t_m − 49°C) and then to wash the filters sequentially under conditions of increasing stringency (t_m − 49°C, t_m − 39°C, and t_m − 23°C). After each washing step, the filters were exposed to x-ray film for 2 hr. Assuming that the t_m value of a heteroduplex molecule decreases by 1.4°C for every 1% base mismatch (27), these t_m values should theoretically allow detection of hybrids with base mismatch percentages of 42, 28, and 16, respectively. The other method employed was to prepare duplicate Southern blots and to hybridize and wash one blot under conditions of relatively low stringency (t_m − 49°C, base mismatch 35%) and the duplicate blot at higher stringency (t_m − 23°C, base mismatch 16%). The stringency of hybridization and washing solutions was calculated from the known G+C content of HTLV-I (53.9%) derived from Seiki *et al.* (24) and the following relationship (29): t_m = 81.5 + 16.6(log M) + 0.41(% G+C) − 0.72(% formamide), in which M is the monovalent salt molarity and (% G+C) is the percentage of guanine plus cytosine residues in the DNA. Accordingly, the hybridization and washing solutions contained 1× to 6× standard saline/citrate (0.195 M to 1.17 M sodium) and 0% to 40% (vol/vol) formamide while incubation temperatures ranged from 37°C to 81°C. Hybridization solutions also contained 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, tRNA at 20 μg/ml, and 10% dextran sulfate. In all experiments, hybridizations were performed for 18 hr in a total volume of 1.5 ml, using 3×10^6 dpm of probe (specific activity approximately 2×10^8 dpm/μg of DNA). Washes were carried out for 2 hr each at the appropriate temperatures.

Heteroduplex Formation/Electron Microscopy. Electron microscopic heteroduplex analysis of cloned DNAs was performed as described (30, 31). When melting analyses were performed, only formamide concentrations in the hyperphase and hypophase were adjusted to maintain isodenaturing conditions (31, 32). Formamide concentrations in the hyperphase typically ranged from 30% to 60% formamide. The effective temperatures (which are expressed as $t_m - \Delta t$, in which Δt is the difference between t_m and the temperature at which the heteroduplex was mounted for microscopy) for 30%, 40%, 50%, and 60% formamide hyperphases were t_m − 39°C, t_m − 32°C, t_m − 25°C, and t_m − 18°C, respectively. Contour lengths were measured from actual micrographs by using a digital length calculator (Numonics). The sizes of cloned inserts were detemined separately in gel electrophoresis experiments.

RESULTS

Comparison of Restriction Maps of HTLV-I and HTLV-II. The restriction enzyme maps of the cloned proviruses λMO15A (HTLV-II), λ23-3 and λCH-1 (HTLV-I), and λMC-1 (HTLV-Ib) are shown in Fig. 1. They are all full-length, and together, they represent examples of all molecular clones of HTLV reported to date.

Southern Blot Analysis. Fig. 2 examines the relative binding (homology) of restriction fragments of the HTLV-I and HTLV-Ib proviruses to full-length HTLV-I and HTLV-II probes. The expected *Xho* I/*Sst* I internal fragments making up the entire genome of each provirus are seen in lanes A_{1-3} (HTLV-I probe, high stringency) and in lanes B_{1-3} (HTLV-II probe, low stringency), indicating that HTLV-I and HTLV-II share substantial homology throughout their genomes. However, it is apparent even at the lowest stringency (t_m − 49°C) that the HTLV-II probe is least homologous to genomic fragments representing most of the envelope region of HTLV-I and HTLV-Ib (1.3-kbp fragment for λMC-1 and 0.7-kbp fragments for λCH-1 and λ23-3). This lesser degree of homology between HTLV-II and HTLV-I/HTLV-Ib in the envelope region becomes more apparent with increasing stringency (lanes C_{1-3} and D_{1-3}). Conversely, the fragments representing the pX plus LTR regions (1.7-kbp fragment of λMC-1 and 2.2-kbp fragments of λCH-1 and λ23-3) are highly homologous between HTLV-II and HTLV-I/HTLV-Ib. The fragments from the *gag/pol* region of the proviruses (4.4-kbp band of λMC-1 and 5.6-kbp bands of λCH-1 and λ23-3) appear to be, overall, of intermediate homology.

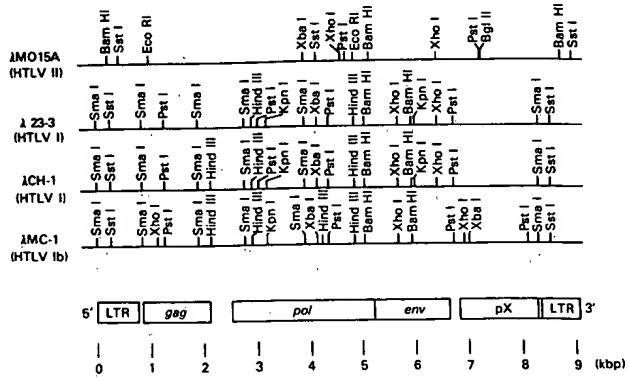

FIG. 1. Restriction endonuclease maps of cloned HTLV proviruses. Genomic regions corresponding to LTR, *gag*, *pol*, *env*, and pX are drawn to scale according to the published nucleotide sequence of an HTLV-I isolate (24). Two *Bgl* II sites in the 5' end of λMO15A are not shown. kbp, Kilobase pairs.

To distinguish between sequence homology in pX versus LTR regions, further experiments were performed using λMC-1 DNA because it contains the restriction sites *Pst* I and *Sma* I dividing pX and LTR fragments (Fig. 1). Fig. 3*A* again demonstrates that the region of the greatest homology between HTLV-Ib and HTLV-II is in pX–LTR (1.7-kbp band) and least homology is in the envelope (1.3-kbp band). The 1.0- and 4.4-kbp fragments representing LTR/*gag* and *gag/pol* regions, respectively, are of intermediate homology. Fig. 3*B*, however, shows that the pX region (1.4-kbp band, lanes A and B; 4.5-kbp band, lanes C and D) exclusive

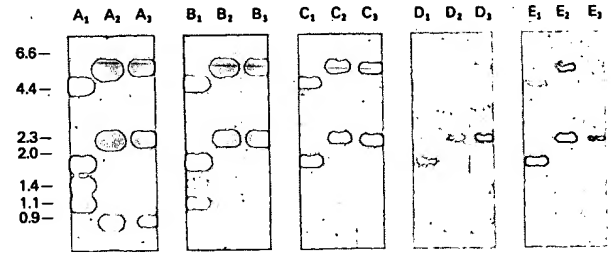

FIG. 2. Hybridization of HTLV-I or HTLV-II probes to Southern blotted restriction fragments of HTLV-I and HTLV-Ib (0.7% agarose gel, 1 μg of DNA per lane). Lanes 1, 2, and 3 contain λMC-1, λCH-1, and λ23-3 DNA, respectively, digested with *Sst* I plus *Xho* I. Lanes A_{1-3} were hybridized to the p23-3i probe (HTLV-I) and lanes B_{1-3} through E_{1-3} to the λMO15Ai probe (HTLV-II). Lanes A_{1-3} (HTLV-I probe at high stringency, t_m − 23°C) show the expected internal restriction fragments for HTLV-I and HTLV-Ib. Lanes B_{1-3} through D_{1-3} were hybridized at low stringency (t_m − 49°C) and then washed sequentially at low (t_m − 49°C), intermediate (t_m − 39°C), and high (t_m − 23°C) stringency (lanes B, C, and D, respectively). Lanes E_{1-3} are duplicate blots which were hybridized at high stringency (t_m − 23°C) and then washed at high stringency (t_m − 23°C). The bands corresponding to the pX plus LTR regions of λMC-1 (1.7 kbp) and of λCH-1 and λ23-3 (2.2 kbp) are most highly conserved as stringency increases, whereas the bands corresponding to the envelope fragments (1.3 kbp for λMC-1 and 0.7 kbp for λ23-3) are least highly conserved.

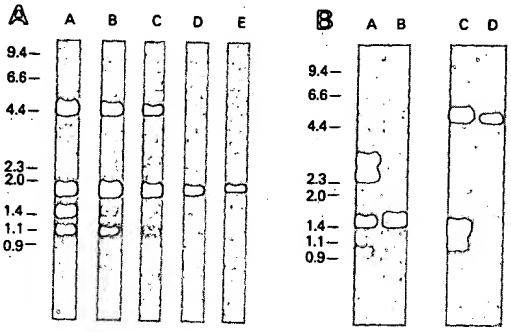

FIG. 3. (*A*) Hybridization of HTLV-I (p23-3i probe, lane A) and HTLV-II (λMO15Ai probe, lanes B–E) to λMC-1 DNA digested with *Sst* I plus *Xho* I. Hybridization and washing conditions for each of the lanes A through E are exactly as described for Fig. 1. The 1.7-kbp band corresponding to the pX plus LTR fragment is most intense, and the 1.3-kbp band corresponding to the envelope region is least intense. The bands representing LTR plus *gag* (1.0 kbp) and *gag* plus *pol* (4.4 kbp) are of intermediate intensity. (*B*) Hybridization of HTLV-I (p23-3i probe, Lanes A and C) and HTLV-II (pMO-3i probe, lanes B and D) to λMC-1 DNA digested with *Sst* I plus *Pst* I (lanes A and B) or *Sst* I plus *Sma* I (lanes C and D). Hybridization and washing were done at high stringency (t_m − 23°C). The intense bands at 1.4 kbp in lane B and at 4.5 kbp in lane D represent hybridization of the HTLV-II probe to the pX region of HTLV-Ib exclusive of LTR and *pol*.

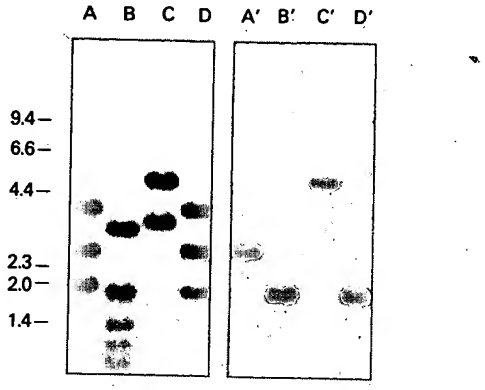

FIG. 4. Hybridization of HTLV-II (pMO-4i probe, lanes A–D) and HTLV-I (p23-3i probe, lanes A′–D′) to Southern blotted restriction fragments of HTLV-II (λMO15A). Lanes A and A′, *Sst* I plus *Xho* I; lanes B and B′, *Sst* I plus *Bgl* II; lanes C and C′, *Sst* I plus *Xba* I; lanes D and D′, *Sst* I plus *Pst* I. Hybridization and washing were each performed under conditions of high stringency (t_m − 23°C). Lanes A–D demonstrate the expected bands for HTLV-II as depicted in Fig. 1. However, hybridization of the HTLV-I probe (lanes A′–D′) to blotted fragments of HTLV-II was most intense in the 3′ end of HTLV-II, corresponding to its putative pX region (see 1.7-kbp *Sst* I/*Bgl* II and 1.7-kbp *Sst* I/*Pst* I bands in lanes B′ and D′). Intermediate intensity hybridization occurred in the 5′ half of HTLV-II (3.6-kbp *Sst* I/*Sst* I fragment in lane A′), and no hybridization was observed between the HTLV-I probe and the presumed *pol–env* region of HTLV-II (see 1.8-kbp *Xho* I/*Xho* I bands in lanes A and A′ and 2.5-kbp *Pst* I/*Pst* I bands in lanes D and D′ for comparison).

of the LTR is most homologous between HTLV-II and HTLV-Ib. The LTR bands at approximately 0.5 kbp (*Pst* I) and 0.4 kbp (*Sma* I), still barely visible on the original radiograph, indicate a lesser but nonetheless still significant degree of homology in this region as well. The preservation of strong bands in the pX region at hybridization conditions of highest stringency (t_m − 23°C) suggests that at least part of the pX region has 85% or greater homology between HTLV-II and HTLV-I/HTLV-Ib. The intensity of hybridization is not due to the presence of G+C-rich sequences in this region, since it is known from the sequence data of Seiki *et al.* (24) that the G+C content of the pX region of HTLV-I (54.8%) is not appreciably greater than for the remainder of the genome (53.9%).

These data thus demonstrate that an HTLV-II probe hybridizes most strongly to HTLV-I/HTLV-Ib in the pX region but also, at somewhat lesser stringency, to LTR, *gag*, *pol*, and *env*. In order to determine which regions within the HTLV-II genome are homologous to these HTLV-I and HTLV-Ib restriction fragments, a probe containing the full-length HTLV-I genome (p23-3i) was hybridized to blotted HTLV-II (λMO15A) fragments. As shown in Fig. 4, the 1.7-kbp portion of HTLV-II closest to its 3′ end (*Bgl* II/*Sst* I and *Pst* I/*Sst* I bands in lanes B′ and D′, respectively) is most homologous to HTLV-I, and the region immediately 5′ to this is least homologous. Since the nucleotide sequence of HTLV-II is not available, precise assignment of LTR, *gag*, *pol*, *env*, and pX regions of the genome cannot be made. However, these data would indicate that the genomic organization of HTLV-II is similar to that of HTLV-I, with the 3′ end of HTLV-II, which hybridizes most strongly to HTLV-I, representing pX. The area of least homology would include the envelope region immediately 5′ to pX (*Xho* I/*Xho* I band at 1.8 kbp, lane A), and the region of intermediate homology would consist of *gag–pol* (e.g., *Xho* I/*Sst* I band at 3.6 kbp, lane A). We have confirmed this sequence homology between the pX region of HTLV-I and the 3′ end of HTLV-II by using for a probe a pX-specific sequence of HTLV-I (data not shown) and by electron microscopy (see below).

Electron Microscopic Heteroduplex Analysis. To gain further insight into the regions of homology between HTLV-I and HTLV-II, we employed an alternative method, heteroduplex analysis/electron microscopy, using λ23-3 (HTLV-I) and λMO15A (HTLV-II). Because no known single-cut enzyme could be used to release the MO15A genome intact from its cloning vehicle, the heteroduplexes were prepared between the two viral inserts in λ. Since the two vectors are different derivatives of λ (λJ1 and λgt*WES*·λB), it was possible to distinguish between heteroduplexes and homoduplexes. In spreads prepared at lowest stringency, 30% formamide (t_m − 39°C), a small number of heteroduplex molecules showed complete homology in the insert DNA (Figs. 5 *A* and *a* and 6*A*). However, in 94% of the heteroduplexes mapped, a small substitution of approximately 0.6 kilobases (kb) in the *env*–pX region was present (Figs. 5 *B* and *b* and 6*A*). This substitution appeared most consistently at or near the *env*–pX junction. Of lesser frequency and size were substitutions present in the *pol* and *gag* genes.

In spreads from 40% formamide (t_m − 32°C), the envelope substitution increased in size to cover most of the *env* gene (Figs. 5 *C* and *c* and 6*B*). However, there frequently were one or more small regions of homology in the middle of this substitution. Substitutions in the *pol* and *gag* genes were also more frequent, but strikingly, the pX region demonstrated little or no divergence.

At higher concentrations of formamide (50% and 60%, t_m − 25°C and t_m − 18°C, respectively), the substitutions in all regions enlarged such that whole segments of DNA melted out through the LTRs. Heteroduplexes within the homologous λ arms became rare. However, the region of homology that appeared with greatest frequency mapped in pX (Fig. 5 *D* and *d*).

DISCUSSION

HTLV represents a family of related human retroviruses. Of the many members of the HTLV family that have been characterized to date, two major subgroups have been found, HTLV-I and HTLV-II, along with a variant of HTLV-I des-

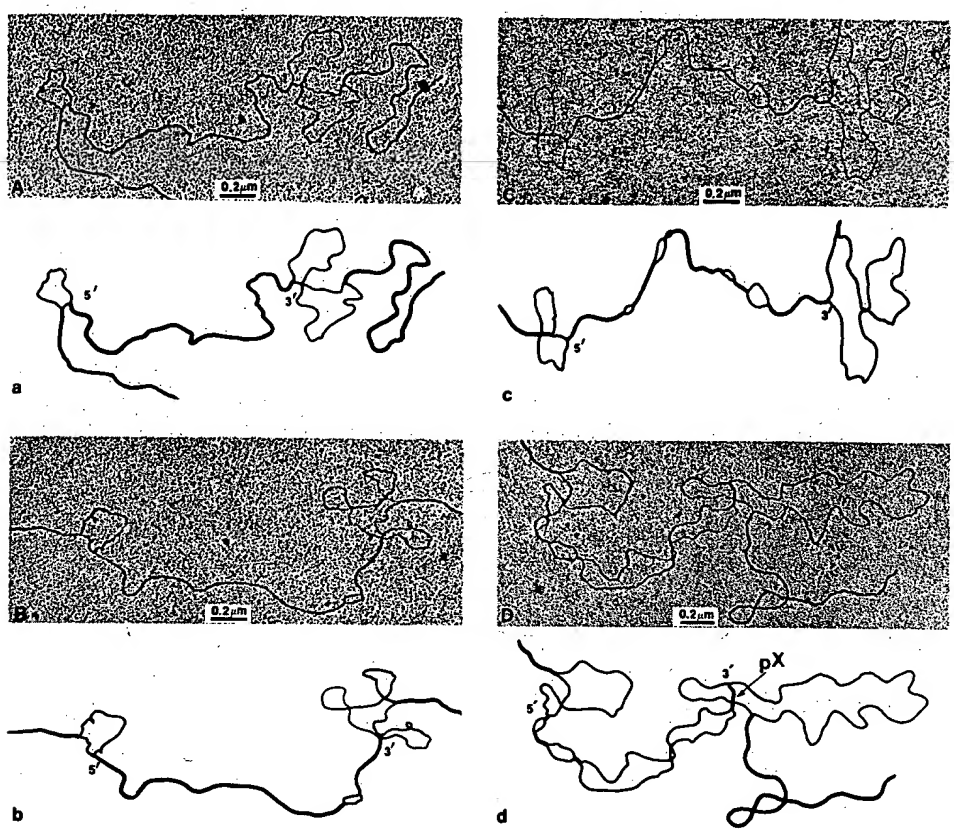

FIG. 5. Heteroduplex analysis of HTLV-I (λ23-3) and HTLV-II (λMO15A) at varied stringencies. (*A* and *B*) 30% formamide (t_m − 39°C); (*C*) 40% formamide (t_m − 32°C); and (*D*) 60% formamide (t_m − 18°C). Actual heteroduplexes are shown in *A*, *B*, *C*, and *D*, and interpretive drawings in *a*, *b*, *c*, and *d*.

ignated HTLV-Ib (15). HTLV-I has been closely linked to a human malignancy (adult T-cell leukemia, ATL) (3, 6–9) and both HTLV-I and HTLV-II have been shown to acutely transform primary cells *in vitro* (4, 8, 21–23), despite lacking an oncogene. The nucleotide sequence of HTLV-I has been determined (24), and from this it is known that the overall genomic organization of HTLV-I is similar to that of other retroviruses, containing LTR, *gag*, *pol*, and *env* regions. However, HTLV-I contains in addition a region identified as pX, which lies 3′ to the envelope sequence and whose function is unknown. This pX region is reported to contain four open reading frames, which potentially can encode proteins of 10,000, 11,000, 12,000, and 27,000 daltons, although none of these putative protein products have as yet been identified (24).

The nucleotide sequence for HTLV-II is not available, but from Southern blot (17, 18) and liquid hybridization (13) analyses it is clearly distinct from HTLV-I. Here, we have shown by two different methods of analysis that there is, surprisingly, substantial nucleotide sequence homology between HTLV-I and HTLV-II throughout the entire length of the genomes, but especially in pX. Under conditions of relatively low stringency (t_m − 49°C, expected base mismatch ≤35%), an HTLV-II probe bound to all internal fragments of HTLV-I (Fig. 2, lanes B_{1-3}, and Fig. 3*A*, lane B), and under similar conditions, electron microscopy showed complete or near complete duplex formation between the molecules (Figs. 5 *A* and *B* and 6*A*). At higher stringency (t_m − 13°C to 23°C), both heteroduplex techniques revealed that the region of greatest homology consisted of the pX region of HTLV-I and a similar 1.7-kbp sequence near the 3′ end of HTLV-II (Figs. 4 and 5 *D* and *d*). These findings are important because they provide experimental evidence for the existence of a pX region in HTLV-II and, moreover, show that this pX region, compared with the remainder of the HTLV genome, is most highly conserved. Since HTLV-I and HTLV-II share certain biologic properties such as T-cell tropism and the ability to acutely transform primary cells *in vitro*, it is possible that the pX region is involved in these or other functions of biologic importance.

The results reported in this paper also demonstrate homology between HTLV-I and HTLV-II in the LTR, *gag*, *pol*, and *env* regions, although apparently less so than for pX. An important qualification here, however, is that both of these methods evaluate homology between relatively long stretches of DNA, and accordingly, short segments of strong or weak homology between the viral genomes may not be recognized. Furthermore, homology at the protein (amino acid) level may be greater than is indicated by nucleic acid homology.

The results in this paper showing relatively strong conservation in nucleotide sequence homology between HTLV-I and HTLV-II in pX and lesser but still substantial homology in LTR, *gag*, *pol*, and *env* are in disagreement with the report of Chen *et al.* (18). Using subclones of HTLV-II encompassing the entire viral genome, they found no hybridization to genomic DNA from HTLV-I-infected cells under standard (unspecified) conditions. Our studies were performed using cloned HTLV-I and HTLV-II DNA, which facilitates the detection of homologous sequences. Still, we have also observed hybridization of HTLV-II probe to genomic DNA from HTLV-I containing cell lines at moderate stringency (t_m − 28°C, predicted base pair mismatch ≤20%), although the signal intensity is much less than for HTLV-II probe hybridized to DNA from HTLV-II-infected cells (unpublished observations). It is likely that different conditions of hybridization, including activity of probes, stringency of hybridization, and amount and HTLV-I content of genomic DNA, explain the differences between our findings.

Finally, serologic studies from our laboratory (ref. 3; unpublished data) and others (33) have shown that various Old World primates have serum antibodies reactive with HTLV

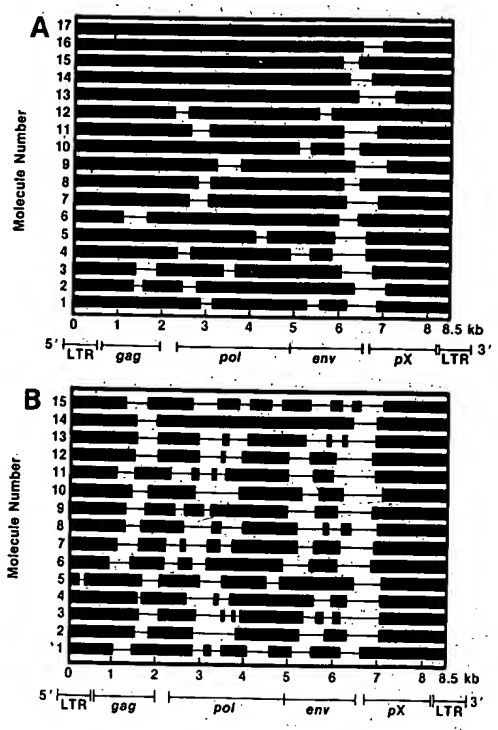

FIG. 6. Bar graph demonstrating the sequence homology between HTLV-I (λ23-3) and HTLV-II (λMO15A) genomes determined from electron microscopic heteroduplex mapping studies. (*A*) 30% formamide (t_m − 39°C); and (*B*) 40% formamide (t_m − 32°C). The horizontal axis in *A* and *B* represents the HTLV-I genome as determined from the nucleotide sequence analysis of Seiki *et al.* (24). The vertical axis designates the particular heteroduplex molecule (HTLV-I·HTLV-II) examined in the mapping studies. Solid bars represent regions of sequence homology, while connecting lines indicate regions of sequence divergence.

proteins. We have recently identified retroviruses from two of these monkey cell lines and found by Southern blot hybridization and restriction enzyme analysis that they are closely related to but distinct from HTLV-I, and from each other (ref. 34; unpublished data). It will be important to make detailed comparisons of these various HTLV-related proviruses to understand more fully their evolutionary relationships and biological properties.

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